Detection and phylogeny of *Wolbachia* in field-collected *Aedes albopictus* and *Aedes aegypti* from Manila City, Philippines

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Abstract

Background: *Wolbachia* is the most common bacterial endosymbiont of arthropods, such as the medically important *Aedes albopictus* and recent reports also detected in *Aedes aegypti.* Our study showed additional support for the presence of wolbachia in *Ae. albopictus* and *Ae. aegypti* using Wolbachia specific markers, *wsp,* and 16S.

Main text: This study collected 12 adult *Ae. albopictus* and 359 *Ae. aegypti* from 183 households in a dengue-prone area, Manila, Philippines, between June and September 2017. *Aedes* larvae (n = 509) were also collected from 17 water containers from 11 households. The DNA of the *Aedes* larvae and adults were screened for the presence of *Wolbachia* using the *wsp* and 16S markers, following optimized polymerase chain reaction (PCR) conditions, and sequenced. Our results showed that 3 out of 359 (0.84%) adult *Ae. aegypti* and 12 out of 12 (100%) adult *Ae. albopictus* were *Wolbachia* positive, whereas all larvae tested negative for *Wolbachia* (0/509; 0%). The *wsp* marker revealed six *Wolbachia*-positive *Ae. albopictus*, whereas the 16S marker showed *Wolbachia* in three *Ae. aegypti* and ten *Ae. albopictus*

Conclusion: Our results suggest that the utilization of two Wolbachia specific markers, *wsp* and *16S* demonstrated Wolbachia presence in individual *Ae. albopictus* and *Ae. aegypti.* The results of the Wolbachia infection in *Ae. albopictus* showed that the detected strains were from either supergroups A and B. Despite the low infection rate of Wolbachia in *Ae. aegypti¸* our results demonstrated the presence of Wolbachia in field-collected *Ae. aegypti* supporting previous studies.

Keywords: *Wolbachia*, dengue, *Aedes aegypti, Aedes albopictus*, Manila City

Background

Wolbachia is a maternally inherited endosymbiotic bacteria, infecting 40% of arthropod species and some filarial nematodes [1-2]. Wolbachia is naturally present in medically important mosquito species, including *Aedes albopictus* [3-5] and *Aedes aegypti* [6-9] inhibiting the replication of arboviral pathogens [10]. Natural populations of *Ae. aegypti* were considered negative for *Wolbachia*, but recent studies have reported both positive [6-9;11-14] and negative [15-16] results for *Wolbachia* in field-collected *Ae. aegypti* among different countries. Due to these varying results, further validation of natural infection of *Wolbachia* in field-collected *Ae. aegypti* is needed.

The presence of natural *Wolbachia* strains in field-collected *Ae. aegypti* is important as it may affect future *Wolbachia* mass release programs, thereby influencing the successful invasion of *Wolbachia* strains from transfected mosquitoes into the natural population. Most studies have found that naturally occurring *Wolbachia* in *Ae. aegypti* are strains that are phylogenetically close to the wAlbB strain, which also infects *Ae. albopictus* [6-8;11]. *Ae. albopictus* is known to be infected with two *Wolbachia* strains, known as wAlbA and wAlbB, which belong to the supergroups A and B, respectively [17]. However, previous studies detecting *Wolbachia* in field-

collected *Ae. aegypti* did not test *Ae. albopictus*, comparing natural Wolbachia strains present in *Ae. aegypti* with *Ae. albopictus* from the same localities at the same time, regarded as naturally infected is important to further characterize and validate natural Wolbachia infection in *Ae. aegypti.*

In this study, we detected *Wolbachia* in field-caught *Ae. albopictus* and *Ae. aegypti* using *Wolbachia*-specific DNA markers (*wsp* and 16S). We also identified and compared the *Wolbachia* strains and supergroups found in *Ae. albopictus* and *Ae. aegypti*. Our study provides further support in the presence of Wolbachia in field-collected mosquitoes.

Main Text

Adults of 12 *Ae. albopictus* and 359 *Ae. Aegypti* were collected using commercially available mosquito light traps from 183 of 428 households randomly selected in Manila City, Philippines from June to September 2017. We found 17 water containers with *Aedes* larvae (*n* = 509) in 11 households. The adults (*n* = 371) and larvae (*n* = 509) were morphologically identified as *Aedes sp.* using a stereomicroscope using the keys of [18]. We extracted DNA using Qiagen AllPrep DNA/RNA micro kit and Qiagen DNA Blood and Tissue DNEasy Kits© (Qiagen, Hilden, Germany) in adult (*n* = 371) and larval (*n* = 509) samples, respectively.

Wolbachia was detected using: *wsp* (610 base pairs) with primer pairs *wsp* 81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3') and *wsp* 691R (5'-AAAAATTAAACGCTACTCCA-3') [16] and 16S specific for *Wolbachia* (850 base pairs) with primer pairs *WolbF* (5'-GAAGATAATGACGGTACTCAC-3') and *Wspecr* (5'-AGCTTC GAGTGAAACCAATTC-3') [19].

For both *wsp* and 16S gene amplification, we followed the PCR protocol published in [7], in a final volume of 10 µl with 1 µl of the genomic DNA. We used the following components for the

68 PCR reaction for both markers: 10x Ex Taq buffer, 25 mM $MgCl_2$, 2.5 mM dNTP, 10 µM forward
69 and reverse primers, 10% dimethyl sulfoxide, and 5 units/µl of Takara Ex Taq™. We included a
70 positive control of a *Wolbachia*-positive *Culex sp.* and negative control of water in each PCR run.
71 PCR products were analyzed in 1.5% agarose gel at 100V for 30 minutes. The criteria for a
72 positive *Wolbachia* test were based on two successful amplifications per marker, *wsp,* and 16S.

73 We assembled and aligned the sequences for each marker using the CodonCode Aligner version
74 1.2.4 and MAFFT version 7. We checked all generated sequences for similarities with reference
75 sequences from GenBank (NCBI, 2016) using Basic Local Alignment Search Tool–Nucleotide
76 BLAST. All *wsp* and 16S, *s*equences were separately analyzed using DNAsp version 6.12.03 [20]
77 to determine the number of haplotypes. We assessed the relationship of the *Wolbachia* strains
78 of our study with representative sequences from different insect hosts by constructing a
79 phylogenetic tree for the *wsp* and 16S sequences using PhyML 3.1 [21].

80 We screened all individual *Aedes sp.* larvae (*n* = 509) and adults (*n* = 371) for *Wolbachia* using
81 the *wsp* and 16S. Larval samples showed no evidence of *Wolbachia* from either marker. However,
82 *Wolbachia* was detected in 15 (4.04%) out of 371 *Aedes sp.* adult mosquitoes based on the *wsp*
83 and 16S. A total of three (0.84%) were *Wolbachia* positive out of 359 *Ae. aegypti*, based on the
84 *wsp* and 12 (100%) out of 12 *Ae. albopictus* (Figure 1), based on the *wsp* and 16S. The *wsp*
85 detected *Wolbachia* in six *Ae. albopictus,* whereas the 16S detected it in three *Ae. aegypti* and
86 ten *Ae. albopictus*. Among these *Wolbachia*-infected *Ae. albopictus,* 4 (26.67%) out of 15 were
87 positive for both *wsp* and 16S. Confirmation of the species identification using the *cox1* revealed
88 that the 12 adult mosquitoes were *Ae. albopictus*, whereas the remaining three were *Ae. aegypti*
89 based on BLAST results with 100% identity match (Table 1).

90 The phylogenetic tree built using the *wsp* (Figure 2 A) revealed two major clades: supergroup A
91 and supergroup B [15]. Two *Ae. albopictus* were infected with *Wolbachia* belonging to
92 supergroup A and four with *Wolbachia* belonging to supergroup B. The haplotype sequences
93 from supergroup A were grouped with the *Wolbachia* type strain A (wAlb A), identified in an *Ae.*
94 *albopictus* host in the USA [17]. In contrast, the sequences from supergroup B clustered with the
95 *Wolbachia* type strains from *Ae. aegypti* (*wAegB*) in the Philippines [6-7] and *Ae. albopictus*
96 (*wAlbB*) in USA [7].

97 In the phylogenetic tree constructed using 16S, 11 out of 13 sequences (84.62%) found in *Ae.*
98 *albopictus* (n = 10) and *Ae. aegypti* (n = 3) were clustered within supergroup A (Figure 2 B). The
99 remaining two (15.38%) found in *Ae. albopictus* were clustered together with the supergroups
100 C, D, E, F, and J from insect hosts such as *Folsomia candida, Mansonella azzardi,* and *Dirofilaria*
101 *immitis*.

102 We found that the *Ae. albopictus* were infected with both supergroups A and B of *Wolbachia*
103 using the *wsp*, widely used for *Wolbachia* strain identification and phylogeny studies [17]. We
104 found more *Wolbachia*-positive *Ae. albopictus* in supergroup B (4/6; 66.66%) than in supergroup
105 A (2/6; 33.33%), as observed in a previous study [22] (China; 631/693; 91.05%). Our results
106 support previous studies, and we observed two *Wolbachia* supergroups in *Ae. albopictus*
107 collected from the same area at the same time.

108 We found a low prevalence of *Wolbachia* in adult *Ae. aegypti*, which is concordant with the
109 previous results of [8] obtained from field-collected *Ae. aegypti* in Florida, USA and those of [7]
110 conducted in Metro Manila, Philippines. In contrast, a high (>50%) prevalence rate was observed
111 in the studies of [9] conducted in Malaysia and [8] conducted in New Mexico, USA. The low
112 *Wolbachia* prevalence rate in *Ae. aegypti* may be due to the low density of the *Wolbachia*

113 endosymbiont in *Ae. aegypti* [7]; this is supported by the previous metabarcoding studies of
114 [8;11-12], which all reported a low number of sequence reads in the *Ae. aegypti* midgut,
115 indicating a low density of *Wolbachia*. Although our results are limited since we did not
116 quantitatively measure *Wolbachia* density, our 40-cycle PCR amplification method adapted from
117 [7] could infer that *Wolbachia*-positive *Ae. aegypti* might be present in Metro Manila, Philippines.
118 Further *Wolbachia* detection studies are thus needed to affirm natural *Wolbachia* infection in
119 field *Ae. aegypti.*

120 We acknowledge the uncertainties of *Wolbachia*-positive *Ae. aegypti* due to the 16S and the
121 conventional PCR method, e.g., the false-positive rate. In light of this information, we were
122 careful to ascertain positive *Wolbachia* in adult *Ae. aegypti*. Thus, we used the primers of [19],
123 which are known to produce fewer false-positive results and negative detections of *Wolbachia*.
124 We also performed repeated PCR tests of our *Wolbachia*-positive samples to ensure successful
125 *Wolbachia* detection as defined in the previous study by [7]. In addition to conventional PCR,
126 tests such as fluorescent *in situ* hybridization and whole genome sequencing can be used to
127 provide strong support for the presence of *Wolbachia* in natural *Ae. aegypti* populations.
128 Despite these limitations, our results provide additional evidence for *Wolbachia* detection in
129 field-collected *Ae. aegypti.*

130 Our results were negative for *Wolbachia* in all the collected *Aedes* larvae (n = 509). One possible
131 reason is that we found only 17 water containers during our field sampling. Since *Wolbachia* is
132 maternally transmitted, all of its offsprings would also be negative for *Wolbachia* if the mother
133 is negative for *Wolbachia*. More *Wolbachia* might be detectable in larval samples if we increase
134 the number of water containers by setting up ovitraps. We also recommend detecting

Wolbachia in representative larval samples from one water container. Future research may also explore the potential horizontal transmission of Wolbachia as observed in the study of [23].

Conclusions

Our study showed the detection of Wolbachia in field-collected *Ae. aegypti* and *Ae. albopictus* in Manila City, Philippines. For *wsp*, we found six *Wolbachia* positive *Ae. albopictus* while the 16S showed three *Ae. aegypti* and ten *Ae.albopictus* positive for Wolbachia. We found 3 out of 359 (0.84%) in adult *Ae. aegypti* and 12 out of 12 (100%) adult *Ae. albopictus* while negative results were found in all larvae (05/509;0%). Our findings may provide additional support for the presence of *Wolbachia* in field-collected *Ae. aegypti* and its ability to invade and persist in the population.

List of Abbreviations

PCR: polymerase chain reaction

DNA: deoxyribonucleic acid

wsp: Wolbachia specific primer

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated and/or analyzed during this study are included in this published article. All newly generated sequences are available in the GenBank database under the Accession Numbers _____. (Accession numbers have not yet been obtained at the time of submission, it will be provided during the review).

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported in part by the Japan Society for the Promotion of Science (JSPS) Grant-in-Aid Fund for the Promotion of Joint International Research [Fostering Joint International Research (B)] under grant number 19KK0107, JSPS Grant-in-Aid for Scientific Research (B) under grant number 21H02206, the JSPS Core-to-Core Program B Asia-Africa Science Platforms under grant number JPJSCCB20190008, and the Endowed Chair Program of the Sumitomo Electric Industries Group Corporate Social Responsibility Foundation.

Author's contributions

MAFR and KW conceptualized and designed the experiment. MAFR and TI collected and identified the adult and larval samples for this study. MAFR and TI performed the *Wolbachia* detection and all the molecular experiments for this study. MAFR, TI and KW performed the data analysis. MAFR and KW wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We are thankful to all the households that participated in our sampling collection of mosquitoes and larvae and to the village officials of Sampaloc, Manila, for their assistance and support. We

appreciate the help of Johanna Beulah T. Sornillo of the Research Institute for Tropical Medicine, Philippines, for her technical assistance in the household sampling design. We want to thank Katherine Viacrusis for her service in the fieldwork and Mr. Micanaldo Francisco for constructing the sampling map. Our deepest thanks to Dr. Thaddeus M. Carvajal, Dr. Divina Amalin, Dr. Mary Jane Flores, Dr. Joeselle M. Serrana, and Jerica Reyes for their valuable suggestions and technical assistance. The authors would like to thank Enago (www.enago.jp) for the English language review. Maria Angenica F. Regilme is a recipient of the Japanese Government (Monbukagakusho) Scholarship from the Ministry of Education, Science, Sport, and Culture of Japan.

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Table 1. Summary of sampling data and Wolbachia detection results

	Total
Surveyed households	428
Households with adult Aedes mosquitoes	183
Households with larval Aedes	11
Collected Aedes mosquitoes	371
Aedes albopictus	12
Aedes aegypti	359
Collected Aedes larvae	509
Wolbachia positive	
Larva	0
Adult Ae. albopictus	12
Adult Ae. aegypti	3
Households with Wolbachia-positive mosquitoes	11

282

283 **Table 2.** A detailed summary of the results of the Wolbachia infection in selected *Aedes*

284 mosquitoes using *wsp* and 16S markers

	House hold	Molecular species identification	Individual code	Haplotypes			
				co x1	***wsp***		**16S** (n = 13)*
					Supergroup	***wsp*** (n = 6)*	
1	SH9	*Ae. albopictus*	40	C1			S7
2	SH27	*Ae. albopictus*	306	C5			S3
3	SH134	*Ae. aegypti*	384	C6			S1
4	SH214	*Ae. albopictus*	425	C1			S6
5	NH43	*Ae. albopictus*	172	C4			S3
6	NH56	*Ae. albopictus* (n=3)	095, 177, 178	C1	A (177) and B (178)	W1 (177) and W2 (178)	S8 (095) and S9 (177)
7	NH107	*Ae. albopictus*	218	C5			S4
8	NH130	*Ae. aegypti*	116	C2			S1
9	NH131	*Ae. albopictus*	240	C5	A	W3	S5
10	NH149	*Ae. albopictus* (n = 3)	258, 259, 260	C1	B (258, 259, 260)	W2 (258, 259, 260)	S6 (258, 259)
11	NH181	*Ae. aegypti*	121	C3			S2
Total number of haplotypes				**6**		**3**	**9**

285

286 * total number of *Wolbachia*-positive mosquitoes

287

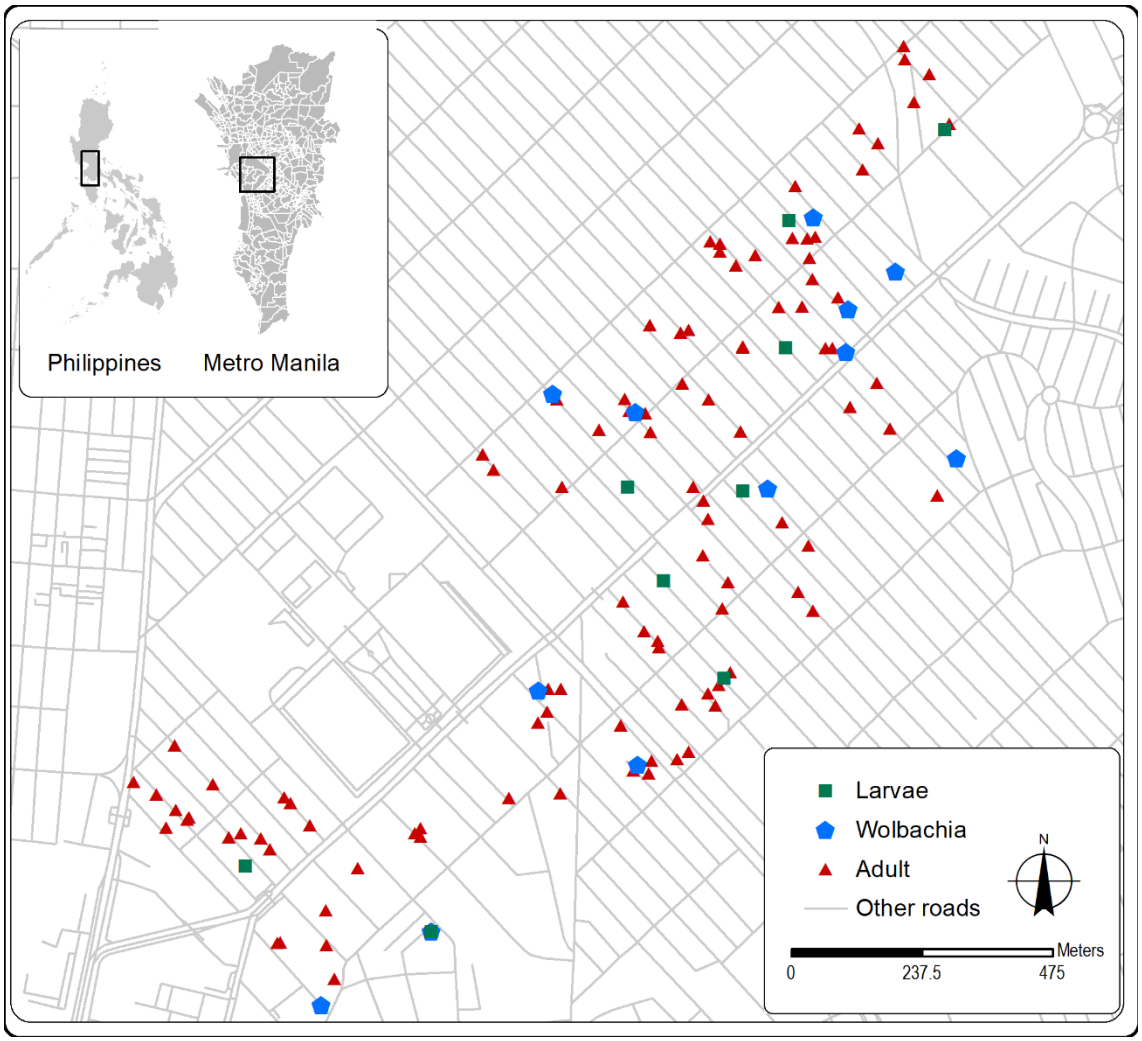

Figure 1. Spatial distribution of *Wolbachia*-positive households (n = 11) out of 428 households surveyed across a 2-kilometer road in highly urbanized area in Manila, Philippines.

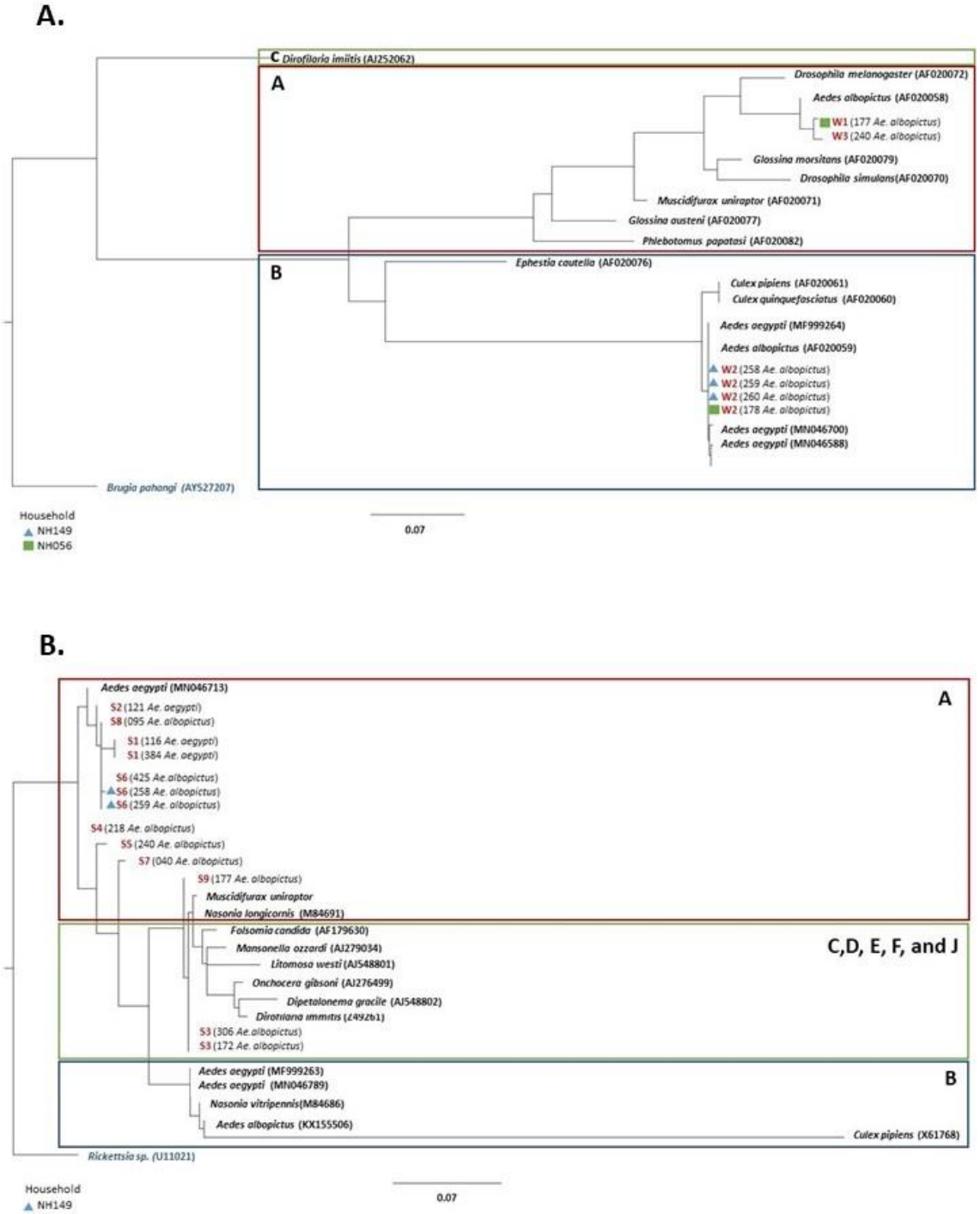

Figure 2. The maximum likelihood phylogenetic tree of *Wolbachia*-infected urban mosquitoes based on the **(A)** *WSP* sequences with reference sequences from supergroups A, B, and C and an outgroup of *Brugia pahangi* **(B)** 16S sequences with reference sequences from supergroups A, B, C, D, E, F, and J and an outgroup of *Rickettsia* sp.